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THE MELANOCYTOTOXIC ACTION OF 4-HYDROXYANISOLE

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Cultured normal mammalian melanocytes exposed to a variety of antioxidants in the presence of millimolar concentrations of 4-hydroxyanisole exhibit dose-dependent modifications of cytotoxicity. While some antioxidants reduced the extent of damage produced by 4-hydroxyanisole, others appeared to increase it. Similar effects were found in a model system using lysis of human erythrocytes as an index of cell damage. Estimations on rat liver microsomes in the presence of tyrosinase and 4-hydroxyanisole showed increased lipid peroxidation only at low substrate concentrations.

The depigmenting action of certain substituted phenols appears to be the result of the selective destruction of melanocytes [1,2]. Possibly this is a result of their conversion to a toxic oxidation product by tyrosinase [3]. The appearance of a new electron spin resonance signal in skin to which 4-hydroxyanisole (4-OHA) had been applied suggests that the toxic metabolite may be a free radical [4,5]. It is possible that the deleterious action of 4-OHA is caused by this metabolite and is a consequence of the initiation of lipid peroxidation and damage to cell membranes. In this paper we report studies made to examine this question. If the lipid peroxidation hypothesis is true it would predict that the disruptive effects of 4-OHA on cultured melanocytes [5] could be modified by the presence of antioxidants, and experiments have been carried out with a number of such agents. In addition, experiments were made to directly test the ability of 4-OHA and tyrosinase system to initiate lipid peroxidation using the generation of malonaldehyde by an in vitro preparation of microsomes as the test system.

MATERIALS AND METHODS

Melanocyte Cultures

Primary cultures were made of melanocytes from the ear skin of young adult guinea pigs of an inbred black strain. Fragments (5 × 5 mm) of ear skin were removed under halothane anesthesia and incubated at 37°C in trypsin in 0.9% saline for 30 min. The dermis was removed and the epidermis washed twice with cold sterile 0.9% saline. The melanocytes and basal keratinocytes were then picked up on flame-sterilized cover glasses by allowing the glass to rest lightly on the skin fragment and transferring the glass to a Perspex culture chamber of the type described by Cruickshank et al [6]. The cover glasses were sealed onto the chambers with beeswax and the culture medium introduced with a sterile syringe. The medium consisted of Eagle's basal medium with Hanks' salts 94.5%, fetal bovine serum 5%, chick embryo extract 0.5%, with penicillin and streptomycin added to 1000 U% of each. The materials were obtained from Flow Laboratories Ltd., Irvine, Ayrshire, Scotland. The cultures were incubated overnight at 37°C with the glass downwards and the chambers then inverted according to the technique of Pruniéras et al [7], and the medium exchanged for new medium which had been gassed with a 95% oxygen/5% carbon dioxide mixture. Because of the greater resistance of the melanocytes to high oxygen tensions, this treatment enables relatively pure melanocyte cultures to be obtained.

4-OHA (Koch-Light Ltd.) recrystallized from benzene was made up in a 0.9% saline solution buffered with 0.1 M phosphate (pH 7.4) (PBS) and added to the culture medium which was then used to replace the medium in the chambers (for details see [5]). The protective agents tested were sodium ascorbate, vitamin E (α-tocopherol glycerol 1000 succinate), reduced glutathionine, menaphthone (vitamin K_3), and butylated hydroxyanisole (BHA). All these agents were made up in phosphate-buffered saline and diluted with standard medium to give final concentrations between 10^{-1} and 10^{-6} M. Ubiquinone was made up in ethanol and diluted with medium to give concentrations between 0.005 mg/ml and 0.1 mg/ml. The antioxidants were added in aliquots to the culture medium. Appropriate controls to which 2-hydroxyanisole, 3-hydroxyanisole, and PBS were added were examined. The assessment of the effect of the antioxidants in each case was made on 30 randomly selected cells after a 30-min incubation at 37°C on the basis of morphologic changes which were classified as follows: cells rounded = slight damage; cells rounded with bleb formation = moderate damage; multiple blebs and fragmentation of dendrites or total disruption of cell = severe damage.

Erythrocyte Suspensions

Suspensions of human erythrocytes were made by resuspending packed cells (group A rhesus positive) as a 10% suspension in 0.9% sodium chloride. A 0.1% solution in saline of mushroom tyrosinase (Sigma Grade II) was dialyzed against normal saline at +4°C for 48 hr to remove potassium before use. All solutions of reagents examined in these experiments were made up in 0.9% sodium chloride. Reaction mixtures were incubated for 1 hr at 37°C. The tubes were then cooled in ice, centrifuged, and the potassium in the supernatant estimated by flame photometry. The results of different experi-

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ments were normalized by expressing them as a percentage of total hemolysis calculated from the potassium released from sonicated control suspensions. Estimation of potassium was preferred to hemoglobin release as an index of cytolysis because of the interference in spectroscopic analysis by the melanoid product of 4-OHA oxidation.

Rat Liver Microsomes

Microsomes were prepared from rat liver homogenates (1:5 w/v in 0.25 M sucrose) by centrifugation at 11,700 × *g* for two periods of 10 min at 0°C, followed by a 40-min centrifugation at 50,000 × *g*. The microsomes were made up in a concentration of 2 μg per ml in 0.15 M potassium chloride. The reaction mixture contained Tris buffer, glucose-6-phosphate, G-6-P dehydrogenase, and NADP as in the method described by Slater [8]. Mushroom tyrosinase (0.1%) prepared as described above and 4-OHA (between 10^{-2} and 10^{-6} M) were added to the test system. The mixtures were incubated in unstoppered flasks at 37°C for 60 min in the dark, the flasks agitated 60 times a minute. After incubation the microsomes were pelleted by centrifugation and resuspended in 4 ml of Tris buffer at pH 7.4. Aliquots of both supernatant and microsomal suspensions were mixed with 10% TCA and butanol extracts were examined spectroscopically. This method of assay for lipid peroxidation is based on the breakdown of lipid peroxidases to give malonaldehyde-like products. The specificity of the reaction is discussed by Slater [8]. The term malonaldehyde is used to mean materials which on heating with thiobarbituric acid give a red color with an extinction maximum at 531 nm. Examination of the absorption spectra showed that when the method was used in the presence of 4-OHA and tyrosinase, a pigment with an extinction maximum at about 450 nm was also formed (Fig. 1). Since this peak was absent when microsomes were omitted it was considered that it might represent a condensation compound formed from the constituents or products of the melanin-generating reaction [4] and the malonaldehyde reaction, thus obscuring the estimation of actual amounts of malonaldehyde generated. For this reason the extinctions at both 450 and 531 nm were recorded together with the sum of the relative extinctions at these wavelengths.

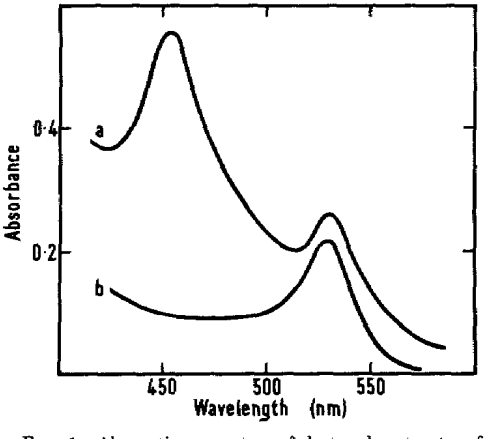

FIG. 1. Absorption spectra of butanol extracts of microsomal reaction mixtures after TCA and thiobarbituric acid treatment. (*a*) With 4-hydroxyanisole and tyrosinase present during incubation; (*b*) control.

RESULTS

Addition of Antioxidants to Melanocytes Exposed to a Standard Toxic Dose of 4-Hydroxyanisole

As previously found, anisole hydroxylated in the 2- and 3-positions was found to be nontoxic in millimolar concentrations, whereas 4-OHA produced moderate or severe cellular damage in cultures less than 7 days old. In older cultures the substance was relatively less effective and this was ascribed to the reduction in tyrosinase activity. The data given in Table I for the effects of a number of antioxidants refer to cultures between 3 and 5 days after seeding. It was found that most of the agents tested, with the exception of vitamin E and ubiquinone, have a protective effect at some concentrations. The effect of ubiquinone was difficult to interpret because of the damaging effect exerted by ethanol alone. The effects of antioxidants seem to fall into three general categories: (1) ascorbate was protective at all dilutions tested, (2) vitamin E failed to protect against 4-OHA at all

TABLE I. *Assessment of morphologic damage to normal melanocytes exposed simultaneously to 10^{-3} M 4-hydroxyanisole and antioxidants*

Antioxidant	Concentration (Molar)	Cellular damage
None	—	Moderate
Ascorbate	10^{-1}	Slight
	10^{-2}	Slight
	10^{-3}	Slight
	10^{-4}	Slight
	10^{-5}	Slight
Menaphthone (vitamin K_3)	10^{-1}	Severe
	10^{-2}	Moderate
	10^{-3}	Moderate
	10^{-4}	None
	10^{-5}	None
Butylated hydroxyanisole (BHA)	10^{-2}	Slight
	10^{-3}	Moderate
	10^{-4}	Moderate
	10^{-5}	Severe
	10^{-6}	Severe
Glutathione (reduced)	10^{-1}	Slight
	10^{-2}	Moderate
	10^{-3}	Moderate
	10^{-4}	Severe
	10^{-5}	Severe
	10^{-6}	Slight
α-Tocopherol glycerol 1000 succinate (vitamin E)	10^{-1}	Severe
	10^{-2}	Severe
	10^{-3}	Severe
	10^{-4}	Severe
	10^{-5}	Severe
	10^{-6}	Severe

dilutions tested, and (3) menaphthone, BHA, and glutathione showed a variation in protective action with alterations in concentration.

Both BHA and reduced glutathione increased cellular damage at 10^{-4} M and 10^{-5} M concentrations but, whereas reduced glutathione like vitamin K had a protective action in greater dilution, butylated hydroxyanisole continued to act synergistically with 4-OHA as the dilution was increased. It is unlikely that this is due to the oxidation of BHA by tyrosinase since in vitro experiments indicate that such oxidation is negligible (Riley, unpublished data). In view of the failure of glutathione to have a protective action at relatively high concentrations it is unlikely that the protection afforded by this agent can be ascribed to tyrosinase inhibition. The clear difference in the effects of vitamin E and ascorbate on the melanocyte system suggested that their influence might be due to actions not directly related to their antioxidant properties. For this reason experiments were performed using an in vitro 4-OHA and tyrosinase system employing the lysis of erythrocytes as the index of membrane damage produced in the presence of various amounts of these two agents.

Effect of Hydroxyanisole Oxidation Products on Erythrocyte Suspensions

The results of these experiments are shown in Figure 2. It was found that 4-OHA alone in 0.5 M concentration did not cause significant hemolysis. However, the addition of ascorbate (*open squares*) or tocopherol (*open circles*) rendered 4-OHA damaging to the erythrocyte membranes. These substances alone had no effect in the concentrations used in the study, and the mechanism whereby they stimulate hemolysis in the presence of 4-OHA is not clear. Tocopherol had a greater damaging effect and this result suggests that the failure of vitamin E to show a protective effect in the melanocyte experiments might, in part, be due to this direct action, particularly in view of the fact that the 45% hemolysis produced by a combination of 4-OHA and tyrosinase was reduced by the addition of both ascorbate (*black squares*) and vitamin E (*black circles*).

Malonaldehyde Production in Suspensions of Rat Liver Microsomes

The results are given in Table II. These show that malonaldehyde pigment with an absorbance maximum at 531 nm is formed in the presence of microsomes. No significant amounts were detected when microsomes were omitted, the slightly raised value in the presence of tyrosinase and 10^{-2} M 4-OHA being due to nonspecific absorbance ascribable to 4-OHA-melanin. Enzyme catalyzed or autooxidation products of 4-OHA also account for the slightly raised extinction values at 450 nm. There is a suggestion that some complex formation occurs when microsomes and tyrosinase are present in the incubation mixture since the absorbance at 531 nm is reduced and that at 450 nm increased; this could mean that the 450-nm material arises by the action of tyrosinase on some intermediate compound in the malonaldehyde reaction. However, a marked rise in the 450-nm material was observed in the absence of tyrosinase. Probably, therefore, the major product giving rise to the extinction at this wavelength is a complex composed of products of the malonaldehyde reaction and derivatives of 4-OHA, and this would be consistent with the results obtained when all the reactants (micro-

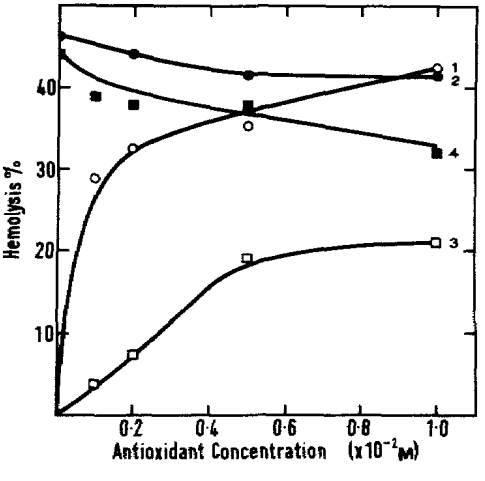

FIG. 2. Effect of increasing concentrations of α-tocopheryl glycerol 1000 succinate (*circles*) and sodium ascorbate (*squares*) on the lysis of human erythrocytes by 5×10^{-4} M 4-hydroxyanisole (O,□) and a mixture of hydroxyanisole and mushroom tyrosinase (●,■).

TABLE II. *Effect of 4-hydroxyanisole and tyrosinase system on microsomal peroxidation*

Microsomes (2.5 ml)	Tyrosinase (1.0 ml)	4-OHA (0.4 ml)	Mean extinction[a]		Sum (531 + 450)
			531 nm	450 nm[b]	
+	0	0	0.232	0.012	0.244
0	+	0	0.006	0.018	0.024
0	0	10^{-2} M	0.002	0.081	0.083
+	+	0	0.072	0.123	0.195
0	+	10^{-2} M	0.097	0.085	0.182
+	0	10^{-2} M	0.057	0.119	0.176
+	0	10^{-3} M	0.114	0.132	0.246
+	0	10^{-4} M	0.234	0.208	0.442
+	+	10^{-2} M	0.083	0.122	0.205
+	+	10^{-3} M	0.137	0.160	0.297
+	+	10^{-4} M	0.276	0.247	0.523

Final volume of reaction mixture = 5.0 ml.

[a] The figures represent the mean values of three separate experiments conducted in duplicate.

[b] Estimated above asymptote to take account of increased absorbance due to melanin in the system.

somes, 4-OHA, and tyrosinase) were present in the system. Taking the sum of the extinctions at 450 and 531 nm as an index of the formation of product, it is evident that at relatively high concentrations the major effect of 4-OHA is to reduce lipid peroxidation but that a pro-oxidant effect is exhibited at low concentrations (10^{-6} M). This effect was enhanced by the inclusion of tyrosinase in the reaction mixture; the total extinction was 0.523 in the presence of 10^{-6} 4-OHA compared to the combined absorbance of the controls of 0.351. The results strongly suggest that lipid peroxidation is stimulated by the presence of oxidation products of hydroxyanisole.

DISCUSSION

The results presented are broadly in agreement with the predictions of the hypothesis outlined at the beginning of this paper insofar as the effects of antioxidants on the damage to melanocytes and red-cell membranes produced by 4-OHA oxidation products are concerned. In view of the free radical scavenger role of these compounds, this may be taken to be evidence that the cell damage resulting from the action of the tyrosinase and 4-OHA mixture is produced by a free radical intermediate. Also the data from the microsome experiments indicate that lipid peroxidation is increased by about 40% in the presence of tyrosinase and low concentrations of 4-OHA. It is possible, therefore, that lipid peroxidation is initiated by an oxidation product of 4-OHA.

Some of the difficulties encountered in demonstrating this mechanism unequivocally arise from the antioxidant nature of hydroxyanisole. It has been shown that 4-OHA is a substrate for tyrosinase [4] but it also has radical scavenger properties due to the electron donor nature of the methoxy group [9], and it is known that antioxidants under certain conditions exhibit pro-oxidant effects [10]. It is possible that the data presented demonstrate the combined effects of these actions. While the results described are by no means conclusive, they are consistent with the suggestion that at least one mechanism whereby the toxic action of 4-OHA (and possibly other similar substituted phenols) is exerted on pigment cells involves the initiation of damage to cell membranes, possibly by a free radical mechanism initiating lipid peroxidation.

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